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Genetic and Phenotypic Variation of Foot-and-Mouth Disease Virus during Serial Passages in a Natural Host[▽]

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Foot-and-mouth disease virus (FMDV), like other RNA viruses, exhibits high mutation rates during replication that have been suggested to be of adaptive value. However, even though genetic variation in RNA viruses and, more specifically, FMDV has been extensively examined during virus replication in a wide variety of in vitro cell cultures, very little is known regarding the generation and effects of genetic variability of virus replication in the natural host under experimental conditions and no genetic data are available regarding the effects of serial passage in natural hosts. Here, we present the results of 20 serial contact transmissions of the highly pathogenic, pig-adapted O Taiwan 97 (O Tw97) isolate of FMDV in swine. We examined the virus genomic consensus sequences for a total of 37 full-length viral genomes recovered from 20 in vivo passages. The characteristics and distributions of changes in the sequences during the series of pig infections were analyzed in comparison to the O Tw97 genomes recovered from serially infected BHK-21 cell cultures. Unexpectedly, a significant reduction of virulence upon pig passages was observed, and finally, interruption of the viral transmission chain occurred after the14th pig passage (T14). Virus was, however, isolated from the tonsils and nasal swabs of the asymptomatic T15 pigs at 26 days postcontact, consistent with a natural establishment of the carrier state previously described only for ruminants. Surprisingly, the region encoding the capsid protein VP1 (1D) did not show amino acid changes during in vivo passages. These data demonstrate that contact transmission of FMDV O Tw97 in pigs mimics the fitness loss induced by the bottleneck effect, which was previously observed by others during plaque-to-plaque FMDV passage in vitro, suggesting that unknown mechanisms of virulence recovery might be necessary during the evolution and perpetuation of FMDV in nature.

Foot-and-mouth disease (FMD) has a high economical impact, affecting domestic and wild cloven-hoofed animal species worldwide (reviewed in references 2, 3, 24, and 46). The etiological agent FMD virus (FMDV) of *Picornaviridae* occurs as seven distinct serotypes and multiple subtypes, reflecting significant genetic and antigenic heterogeneity. In the field, this heterogeneity is reflected by the lack of cross-protection even between intraserotype variants (2, 3, 46).

VP1 (1D), the highly variable FMDV capsid protein with roles in virus entry, immunity, and serotype specificity, has been the subject of extensive comparative sequence analysis (reviewed in reference 22). These studies have shown cocirculation of FMDV genotypes in single outbreaks, with genotypes usually grouping into geographically and genetically distinct lineages (less than 15% nucleotide differences) known as topotypes (41). With the expansion of FMDV genomic databases, however, evidence is accumulating for the inadequacy of VP1 analysis alone for epidemiological studies and for the importance of recombination in FMDV evolution (4, 20, 23).

The selective forces at work during the emergence of FMDV populations in nature are likely to be influenced by specific epidemiological and immunological aspects of host-virus interaction as well as the quasispecies composition of the viral population. Many important questions, including those regarding the significance of high mutation rates in adaptive virus evolution, of Darwinian selection in diversification of viruses with short infection cycles, and of genetic drift as a mechanism for FMDV evolution, remain unanswered. Similarly, there is no knowledge of the limits within which a highly variable pathogen, such as FMDV, can accumulate genomic changes and still reproduce the disease in the natural host and spread in the natural environment. Very few studies have been published regarding FMDV-natural host interactions at the genetic level (5, 6, 47, 49). No studies have been conducted to examine FMDV evolution during replication in the natural host, and very few evolutionary analyses have examined genomic regions other than those corresponding to VP1 or its precursor, P1 (4, 27). Paradoxically, the few experimental studies conducted with natural isolates suggest extreme constrains for 1D variation (5, 6) and loss of fitness during passages in natural hosts (1, 22, 46). In fact, enhanced mutagenesis experiments have shown infectivity loss for a number of RNA viruses, including FMDV, lymphocytic choriomeningitis virus, and Hantavirus (18, 31, 32, 33, 38, 42), suggesting that critical variability thresholds that may explain the restrictions for change observed in vivo exist. However, the characteristics and

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TABLE 1. FMDV passage in vivo and in vitro

Pig no.	Pig passage	Avg $TCID_{50}$ ($n = 4$)[a]	Observations	BHK-21 cell passage	Avg $TCID_{50}$ ($n = 4$)[a]
48	T00	5.92 ± 0.54	Injected to produce stock	P1A	5.8 ± 0.51
50	T00	5.81 ± 1.1	Injected to produce stock	P1B	5.8 ± 0.39
483	T0	7.30 ± 0	Injected, in contact with two naive pigs[b]	P2A	7.6 ± 0.54
484	T1	7.13 ± 0.67		P2B	6.3 ± 0.51
485	T1	7.97 ± 0.17		P3A	6.8 ± 1.55
486	T2	5.79 ± 0.3		P3B	5.8 ± 1.2
487	T2	7.58 ± 0.39		P4A	6.6 ± 0.51
488	T3	5.29 ± 0.86		P4B	6.6 ± 0.72
489	T3	3.5 ± 0.9		P5A	6.5 ± 0.5
490	T4	7.09 ± 0.46		P5B	6.5 ± 0.22
491	T4	8.34 ± 1.04		P6A	6.8 ± 0.72
492	T5	5.8 ± 1.0		P6B	6.6 ± 0.5
493	T5	5.93 ± 0.37		P7A	6.1 ± 0.05
494	T6	7.75 ± 0.05		P7B	6.8 ± 0.37
495	T6	6.46 ± 0.16		P8A	6.3 ± 0.54
496	T7	5.3 ± 0.83		P8B	7.3 ± 1.55
497	T7	7.8 ± 0.63		P9A	6.3 ± 0.27
498	T8	6.74 ± 1.4		P9B	7.3 ± 1.04
499	T8	5.35 ± 0.78		P10A	6.6 ± 0.3
725	T9	3.07 ± 0.27		P10B	6.6 ± 0.39
726	T9	7.07 ± 0.44		P11A	6.8 ± 0.51
4500	T10	5.21 ± 3.9		P11B	6.8 ± 0.27
5008	T10	1.84 ± 1		P12A	7.5 ± 0.05
5009	T11	4.02 ± 0.45		P12B	6.1 ± 0.17
5010	T11	6.85 ± 0.27		P13A	6.5 ± 0.54
5011	T12	5.57 ± 0.22		P13B	6.8 ± 1.04
5012	T12	6.41 ± 0.89		P14A	7.1 ± 0.72
5013	T13	8.3 ± 0.17		P14B	6.0 ± 0.39
5014	T13	5.9 ± 0.61		P15A	7.0 ± 0.45
5015	T14	6.21 ± 0.6	Contact transmitted with no clinical signs	P15B	7.0 ± 0.27
5016	T14	<0.8	Contact transmitted with no clinical signs	P16A	7.0 ± 0.54
4823	T15	5.85 ± 0.5	Injected with 5015 vesicular fluid[c]	P16B	6.8 ± 0.61
4824	T16	5.75 ± 0.72	In contact with 4823	P17A	7.3 ± 0.16
4825	T17	6.92 ± 0.51		P17B	6.6 ± 0.51
4826	T17	<0.8		P18A	7.0 ± 0.89
5184	T18	<0.8	Contact transmitted with no clinical signs	P18B	6.8 ± 0.44
5185	T18	1.97 ± 1.2	Contact transmitted with no clinical signs	P19A	7.3 ± 1.04
5005	T19	3.42 ± 1.55	Injected with 5185 vesicular fluid[d]	P19B	6.6 ± 0.22
5006	T20	<0.8	Contact transmitted with no clinical signs	P20A	7.1 ± 0.17
5007	T20	<0.8	Contact transmitted with no clinical signs	P20B	6.8 ± 0.05

[a] Titers are expressed as $\log_{10}$ numbers of of $TCID_{50}$/ml. Titer values represent the means and standard deviations for four different vesicles from the same pig or four replicates of titration of supernatant from the same cell culture.
[b] Pig 483 was inoculated i.d. with $10^{6.37}$ $TCID_{50}$ from original viral stock T00.
[c] Pig 4823 was inoculated i.d. with $10^{5.7}$ $TCID_{50}$ from pig 5015 vesicular fluid.
[d] Pig 5005 was inoculated with $10^{1.15}$ $TCID_{50}$ from pig 5185 vesicular fluid.

boundaries of those limits in genetic variation and phenotypic expression remain unknown.

Here, we analyzed genetic changes in full-length FMDV genomes during serial passages of O Taiwan 97 (O Tw97) virus in pigs and in BHK-21 cells. Originally isolated from pigs during an FMD outbreak, O Tw97 virus exhibits rapid spread and high virulence in pigs (13, 21, 50). New FMDV genetic variants with altered pathogenicity in pigs and the rapid replacement of the original consensus sequence by new variant genotypes with acquired mutations, mostly outside the capsid coding region P1, were observed. The data indicate rapid accumulation of nucleotide substitutions and fitness loss, suggesting bottleneck transmission effects. Fixation of amino acid changes in nonstructural proteins (NSPs) likely resulted in deleterious effects for virus biology, leading to the establishment of a subclinical infection that resembles the carrier state described for cattle (2, 39, 46). Furthermore, we found significant differences in evolution parameters between in vivo- and in vitro-passaged virus, reflecting differences in selective pressures operating on virus populations, expressed as differences between numbers of synonymous and nonsynonymous substitutions, frequencies of transitions and transversions, and levels of tolerance for changes in specific viral proteins.

MATERIALS AND METHODS

Viruses. O Tw97 virus was obtained from the Animal and Plant Health Inspection Service, USDA, as a 10% swine epithelial tissue homogenate and was completely sequenced (GenBank no. AY593835). For stock virus production, two pigs (no. 48 and 50) were inoculated with the supernatant of the epithelial homogenate (0.5 ml; $10^{5.7}$ 50% tissue culture infectious doses [$TCID_{50}$]/ml) by the intradermal (i.d.) route. Within 24 h after inoculation, both animals developed high fevers and generalized lesions with large vesicles on the feet and snout, symptoms consistent with previous descriptions of O Tw97 virus infection (14, 50). Vesicular fluid was collected from independent lesions and pooled to create

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a viral stock, T00. The T00 stock was titrated and the FMDV genome completely sequenced and then used to infect a pig i.d. in pig passage T0 (Table 1).

Virus titration. Eight $\log_{10}$ serial dilutions of vesicle fluid were used to inoculate BHK-21 cells. After 1 h of virus adsorption, cells were cultivated with 50 μl medium containing 2% fetal calf serum (FCS) at 37°C and 5% CO_2 for 72 h. Cell monolayers were stained with neutral violet, plaques counted, and titers determined using the Reed and Muench method (37) and expressed as numbers of $TCID_{50}$.

Animal experiments. For the serial passage experiment, 4-week-old pigs were randomly paired and housed in containment rooms. One pig, T0 (meaning time zero of infection), was inoculated by the i.d. route with 100 μl of T00 ($10^{6.47}$ $TCID_{50}$/ml) and housed with two recipient pigs (T1 [time 1]) in the same room. When the body temperatures of the T1 pigs reached 104°F or above, the T1 pigs were moved to a clean room with two noninfected pigs (T2 [time 2]). The period of time between the T1-T2 contact and the appearance of vesicular lesions on the feet and/or mouth of any of the T2 pigs is what we define as the "infectious round." The number of cohabitation days for each infectious round differed between passages. This procedure was repeated for every infectious round up to T13, the last round of the transmission-of-infection chain. For each infectious round, when vesicles in donors became evident, vesicle fluid was collected, and the animals were kept in contact with the recipient animals until fever occurred (i.e., T3); then, the animals were culled. For each infected animal, we recorded daily body temperatures and the presence of clinical symptoms.

Sample collection. Vesicular fluids were individually collected with sterile syringes, placed on ice, and transported to the laboratory, where titrations were immediately performed. The remaining volume was stored at −70°C until used for RNA extraction and sequencing. Epithelial tissue from broken vesicles was collected using clean sterile scissors, immersed in cryotubes containing 500 μl of Dulbecco's modified Eagle's medium (DMEM), and immediately frozen at −70°C. Tonsil scrapings and nasal swabs were collected from animals that did not present signs of disease after 26 days in contact with donor animals. This material was used for both reverse transcription (RT)-PCR and virus isolation in BHK-21 cells.

BHK-21 cell culture infections. BHK-21 cells were grown in T25 tissue culture flasks with DMEM containing 5% FCS. Cells were serially passaged 23 times at a concentration of 10^5 cells/ml. Infections were carried out when cells were approximately 95% confluent using a multiplicity of infection of 1 to 10 virus particles per cell from the previous viral passage and cultured in DMEM with 2% FCS. The first passage, P1, was carried out with 0.5 ml of vesicular fluid containing $10^{6.47}$ $TCDI_{50}$s/ml from the T00 stock virus, resulting in a multiplicity of infection of 1 to 10 virus particles per cell. In this case, we define the infectious round as the period of time between the culture inoculation and the detection of a complete cytopathic effect. When the cytopathic effect was complete, the culture was frozen and thawed three consecutive times; the supernatant was clarified by centrifugation at 3,000 rpm for 10 min and fractionated in 1-ml aliquots at −70°C. For the next infectious round, 1 ml of the supernatant was used for infecting BHK-21 tissue cultures in duplicate (P2a and P2b). By repetition of these steps, serial infections of separated lineages (A and B) were carried out up to passages 23A and 23B. Titrations of every infected cell culture supernatant were performed for each passage.

RT-PCR and sequencing. Total RNA was directly extracted from 140 μl of the DMEM-vesicular fluid mixture or from infected cell culture supernatants. Full-length FMDV genome sequences were obtained by RT of the viral genomic RNA, followed by amplification and sequencing of overlapping cDNA fragments spanning the entire viral genome as previously described (4).

Direct DNA sequencing of amplicons derived from a given FMDV isolate yielded a consensus sequence representing the most probable nucleotide for each position of the sequence. This approach prevented analysis of minor sequence variants, polymerase misincorporation errors, and sequencing ambiguities through multiple independent cDNA synthesis, PCR amplification, and direct sequencing events. Due to the quasispecies nature of FMDV populations, polymorphisms were detected in some nucleotide positions. Nevertheless, all positions could be unambiguously assigned to a single dominant nucleotide due to the high degree of redundancy generated by the sequencing strategy.

Sequence analysis. As described previously (4), bases were called from chromatogram traces with the Phred program, which also produced a quality file containing a predicted error probability at each base position. Viral sequences were assembled with the Phrap and CAP3 assemblers. Gap closure was performed as described previously (4). Multiple sequence alignments were performed with the ClustalW (version 1.7) computer program. Analyses of codons and synonymous/nonsynonymous substitution ratios were calculated using the programs SNAP, CodonW (http://www.molbiol.ox.ac.uk/cu/), and codeml (PAML3.14 package), which was also used for statistical evaluation of heterogeneous selection pressures at amino acid sites. For protein analysis, the PRETTY program was used. Protein secondary-structure predictions were performed using the GOR and Pratt computer programs. The codeml program was used to analyze and compare predicted positively selected sites in the FMDV genome under in vitro and in vivo growth conditions. A Bayes Empirical Bayes (BEB) analysis-based codeml model giving the highest probability values (P = 0.001) was chosen for the analysis.

RESULTS

FMDV infections and disease presentation. After i.d. inoculation, fevers and soreness were present within 12 h postinfection, with numerous large vesicles appearing on the feet, nose, and tongue at 24 h postinfection. In some cases, complete prostration accompanied by the loss of hooves was observed. During pig-to-pig contact transmission, animals presented severe signs of disease, including salivation, soreness, and loss of appetite, and large vesicles on the nose, mouth, and feet appeared rapidly postcontact for the first three transmission rounds (T1, T2 and T3). Clinical symptoms gradually decreased in severity as the number of passages increased, and acute disease was not observed in the T15 pig passage. Pig 5016 (T14) did not show a fever during the 26 days of observation, despite the fact that vesicle fluids collected from its two T13 contacts (Table 1) contained $10^{8.3}$ $TCID_{50}$/ml and $10^{5.9}$ $TCID_{50}$/ml (pigs 5013 and 5014, respectively). Pig 5015 (T14) developed mild symptoms of disease; after a significant delay in the onset of a fever, a few small vesicles, containing $10^{6.2}$ $TCID_{50}$/ml on average, were detected. The T15 animals, pigs 5017 and 4822, did not exhibit any clinical signs after 26 days in contact with T14 animals. Infectious virus isolated from tonsil scrapings and nasal swabs collected from these animals had titers in the range of 10^4 $TCID_{50}$/ml, however, indicating that infection had occurred in the absence of clinical disease.

The absence of clinical symptoms and vesicles in T15 animals suggested altered infection potential for T14-derived virus. To asses the infectious potential of T14 viral progeny, 25 μl of vesicle fluid from pig 5015 containing $10^{6.63}$ $TCID_{50}$/ml was i.d. inoculated into pig 4823, which presented generalized FMD at 7 days postinoculation. Virus recovered from pig 4823 vesicular fluid (containing $10^{5.85}$ $TCID_{50}$/ml) was labeled as the T15 passage and was able to transmit disease via contact to a T16 recipient pig, no. 4824, with vesicular fluid containing $10^{5.75}$ $TCID_{50}$/ml. The T17 contact animals, no. 4825 and 4826, exhibited a few small vesicles, but they were able to transmit the disease to T18 pigs 5184 and 5185. Although viral RNA was isolated from both T18 pigs, infection was not transmitted and infectious virus ($10^{1.97}$ $TCID_{50}$/ml) was detected only in a tonsil scraping from pig 5185. Similarly to previous observations for reestablishment of T14 contact transmission, T18 vesicular fluid (150 μl containing $10^{1.97}$ $TCID_{50}$/ml) was infectious when inoculated i.d. into a naïve pig, no. 5005. Acute clinical disease was observed, although vesicle virus titers were low ($10^{3.42}$ $TCID_{50}$/ml). T20 pigs 5006 and 5007 were free of clinical signs of infection and failed to transmit infection to T21 pigs, thus ending viral transmission.

The interruption of contact FMDV transmission (Table 1) observed here was accompanied by reductions in the numbers and sizes of vesicular lesions and by a dramatic reduction of virus present in vesicular fluid, indicating a gradual loss of virulence of O Tw97 FMDV on repeated pig passage.

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TABLE 2. Progressive substitutions of dominant nucleotides in the consensus O Tw97 genome sequence during pig passage

Pig designation[c]	Infectious round[d]	Nucleotide in indicated position and region																											
		5′ UTR[a]											3′ UTR	L[pro]						P1									
		82[b]	155	403	474	480	524	541	818	844	955		8096	1087	1411	1444	1522	1573	1611	2290	2793	2860	2881	2890	3040	3724	3763		
Parental	O Tw97[e]	T	T	G	A	G	A	C	T	A	A		T	A	G	A	T	T	A	A	A	C	T	C	A	T	A		
48	T00v1																												
48	T00v2																												
48	T00v3																												
50	T00v1																												
50	T00v2																												
50	T00v3																												
483	T0																												
484	T1																							T					
485	T1								C																G				
486	T2																								G				
487	T2				G										A			C											
488	T3				G										A														
489	T3														A														
490	T4				G										A														
491	T4				G										A														
492	T5				G										A														
493	T5				G										A														
494	T6				G										A														
495	T6				G						G				A														
496	T7														A														
497	T7		C					T						G	A														
498	T8		C		G									G	A														
499	T8		C		G										A														
725	T9		C		G		G							G	A														
726	T9		C					T						G	A														
4500	T10		C											G	A														
5008	T10		C												A														
5009	T11		C												A														
5010	T11		C											G	A							T							
5011	T12		C											G	A							T							
5012	T12		C		G									G	A							T							
5013	T13		C		G									G	A							T	C						
5014	T13		C		G									G	A							T							
5015	T14		C	A	G									G	A	G					G	T							
5016	T14																												
4823	T15 (14-I)		C																										
4824	T16		C											G	A						G							G	
4825	T17		C							G				G	A	G	C				G	T							
4826	T17		C		G	A				G				G	A	G											C		
5184	T18		C											G	A														
5185	T18		C	A	G					G					A														
5005	T19 (18-I)	C	C		G					G				G	A	G					G	T							
5006	T20	C	C		G					G			G	G	A	G	C		C	G	G	T							
5007	T20		C		G					G					A						G	T							

[a] Genomic regions include UTRs and precursor protein-coding regions.
[b] Specific position number in the full-length O Tw97 genome (GenBank accession no. AY593835).
[c] Pig identification number.
[d] The infectious round represented by each pig number is shown, followed by the nucleotide substitutions detected in the corresponding consensus sequence. Empty spaces indicate no change of nucleotides with respect to the parental O Tw97 sequence.
[e] The O Tw97 row indicates the nucleotides assigned for the parental consensus O Tw97 sequence.

Characterization and distribution of nucleotide substitutions in the consensus FMDV genome sequence. Initial rounds of i.d. virus inoculation resulted in full conservation of the consensus sequence as confirmed by genome sequencing of T00 and T0 viruses collected from several vesicles (48T00v1, 48T00v2, 48T00v3, 50T00v1, 50T00v2, 50T00v3, and 483 T0 in Table 2). In contrast, contact pig passages induced both transitory and permanent mutations in the viral genome as early as the first passage (Table 2).

During pig passages, 11 out of 48 nucleotide changes were located in the untranslated regions (UTRs) and 37 were detected in the coding region (open reading frame [ORF]). Of these, approximately 76% were silent substitutions, with only nine (24%) resulting in an amino acid substitution (Tables 3 and 5). Eight nucleotide changes were located in the structural protein (SP)-coding region, with two of them occurring in the VP1-coding region (Tables 2 and 3). Changes in VP1 (1D) were transitory. The NSP-coding region contained 29 of the total 37 nucleotide substitutions located in coding regions (Tables 2, 3, and 5). The 2C region seemed most prone to muta-

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TABLE 2—*Continued*

Nucleotide in indicated position and region																							
P2													P3										
4015	4252	4426	4438	4469	4779	5002	2032	5107	5257	5276	5291	5656	5986	6283	6311	6364	6641	7076	7183	7375	7561	7792
C	T	G	A	G	C	C	C	A	C	A	C	T	G	T	C	T	G	A	C	C	C	C
						T																
						T																
	C																					
	C																			T		
A	C																					
A	C															C				T		
A	C																			T		
A	C											C				C				T		
A	C															C				T		
A	C															C				T		
	C															C				T		
	C								T				A							T		
	C												A							T		
	C								T				A							T		
	C												A							T		
	C								T				A							T		
	C												A							T		
	C								T	G			A							T		
	C								T	G			A							T		
	C								T	G			A						G		T		
	C								T	G			A						G		T		
	C								T	G			A						G		T		
	C				A	T			T	G			A						G		T		
	C								T	G			A						G		T		
	C								T	G			A		T				G		T		
													A		T				G		T		
													A		C	T	C		G		T		
	C	A	G						T	G			A		T		C		G	T	T		T
	C								T	G			A						G	T	T		
	C							G	T	G			A		T		C		G	T	T		
	C							G	T	G			A		T		C		G	T	T		
							T		T	G	T		A		T		C		G	T	T	T	
	C							G	T	G			A		T		C		G	T	T		
	C								T	G			A		T		C		G	T	T		

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tion, exhibiting 10 nucleotide changes, followed by the Leader protease (L^{pro}) and the polymerase 3D, with 6 mutations each. Amino acid changes in the host-range-associated protein 3A were not observed during pig passages.

In contrast, for BHK-21 cell culture-passaged FMDV, 35% of the mutations were observed in the SP region, with 4 substitutions in VP1 (1D). All resulted in amino acid changes, with 44% of all amino acid substitutions concentrated in the SP. Interestingly, the host-range-associated protein 3A was also very well conserved (data not shown).

Quantification of nucleotide and amino acid substitutions per round of infection. The average difference between the two viral populations in the same pig passage was 4.9×10^{-4} substitutions per nucleotide. To estimate the rate of fixation of mutation during passages, we considered the infectious round a "time" unit (see Materials and Methods). Based on this, the rate of fixation of mutation during pig passages was 6.4×10^{-4} substitutions per nucleotide per infectious round (Table 4). The accumulation of nontransitory mutations (substitutions per nucleotide per infectious round) with successive passages resulted in significant differences in rates of accumulation of substitutions, ranging from 3.7×10^{-4} substitutions per nucleotide between the parental and first-passage progeny (T0/T1) sequences to 2.4×10^{-3} substitutions per nucleotide between the parental and 20th-passage progeny (T0/T20) sequences (Table 4). However, there is also a tendency for the number of substitutions per infectious round to increase, as shown in Table 4 in the column indicating the numbers of substitutions per nucleotide per infectious round between passages, where the first passages (i.e., T1/T2) show differences ranging from

TABLE 3. Numbers and distributions of nucleotide and amino acid substitutions observed in the O Tw97 genome consensus sequence during passages in vivo and in vitro[a]

Passage group	No. (%) of substitutions for indicated region[b]																							
	L^{pro}		P1								P2						P3							
			VP4		VP3		VP2		VP1		2A		2B		2C		3A		3B		3C		3D	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
In vivo	6 (16)	1 (11)	0	0	1 (3)	0	5 (13)	1 (11)	2 (5)	0	0	0	2 (5)	1 (11)	10 (27)	3 (33)	1 (3)	0	0	0	4 (11)	1 (11)	6 (16)	2 (22)
In vitro	3 (15)	2 (12)	1 (5)	1 (6)	1 (5)	1 (6)	1 (5)	1 (6)	4 (20)	4 (25)	0	0	1 (5)	0	1 (5)	1 (6)	1 (5)	1 (6)	1 (5)	1 (6)	1 (5)	1 (6)	5 (24)	3 (19)

[a] The total numbers of substitutions were 48 in vivo and 22 in vitro. Total numbers (percentages) of substitutions for subgroups are as follows: for UTRs in vivo, 11 (23); for ORFs in vivo, 37 (77); for UTRs in vitro, 2 (9); for ORFs in vitro, 20 (91); for nucleotides in P1 in vivo, 8 (22); for amino acids in P1 in vivo, 1 (11); for nucleotides in P2 in vivo, 12 (32); for amino acids in P2 in vivo, 4 (44); for nucleotides in P3 in vivo, 11 (30) ; for amino acids in P3 in vivo, 3 (33); for nucleotides in P1 in vitro, 7 (35); for amino acids in P1 in vitro, 7 (44); for nucleotides in P2 in vitro, 2 (20); for amino acids in P2 in vitro, 1 (6); for nucleotides in P3 in vitro, 8 (40); for amino acids in P3 in vitro, 6 (37).

[b] nt, nucleotide; aa, amino acid.

1×10^{-4} to 6×10^{-4} substitutions per nucleotide per infectious round but the last passages (i.e., T18/T19) show differences ranging from 8×10^{-4} to 1.7×10^{-3}. Quantification of the number of amino acids changed in the 2,322 amino acids of the complete polyprotein showed an average of 1.1, or a total of 4.6×10^{-4}, substitutions per amino acid per infectious round, with higher rates between passages T13 and T18, concurrent with loss of transmission capability of the disease.

Predicted selective pressures in vivo and in vitro. The distribution and translational significance of the nucleotide substitutions in both in vitro and in vivo populations were significantly different (Fig. 1). Remarkably, 76% and 21% of the substitutions occurred in the third codon position during animal-to-animal and cell culture virus passages, respectively (SNAP and CodonW results not shown). The BEB calculation of probabilities for site classes, implemented for the best-fitted model (M8 [β and ω]) to the nonsynonymous/synonymous rate ratio (d_N/d_S [ω]) for in vivo and in vitro FMDV populations (30) were estimated to be 10 times lower for pig passages (Fig. 1). The log likelihood value (ln$L$) obtained for the best tree under the substitution model that best fit the data set was reasonably close between both groups. However, the estimated branch lengths for the likelihood analysis, which represents the number of nucleotides substituted per codon, were 5.5 times higher for pig passages than for cell culture-passaged FMDV. The transition/transversion rate ratio, corrected for multiple hits (or κ value), was 4.5 times higher for the cell passages (Fig. 1). To further analyze differences attributable to host selection, we estimated and compared BEB analyses of positively selected sites in the FMDV genome under in vitro and in vivo growth conditions. Amino acid replacements Q580/R in VP2 and P1753/S, E1863/Q, and K2008/E in 3CD were predicted ($P \geq 0.05$) positively selected sites during replication in pigs, while for in vitro-replicating virus, the probability was lower and relevant only for positions in 3D (M2108/T and D2321/G).

DISCUSSION

Repeated contact transmission of the highly virulent O Tw97 isolate of FMD resulted in complete attenuation of pathogenicity in pigs, characterized by an asymptomatic infection resembling the FMD carrier state previously described for ruminants but not previously described as occurring in swine (39). This work is the first to correlate decay of infection levels with fixation and accumulation of genetic mutations in in vivo viral quasispecies during serial infections, which could be interpreted as the result of bottleneck transmission and the genetic effect of Muller's ratchet (7, 29). Such a rapid substitution of the consensus sequence has been demonstrated to occur during in vitro clone-to-clone replication of FMDV (17) and other viruses (7, 8, 10, 51), leading to a detrimental accumulation of mutations. In the case of plaque-to-plaque transfers; however, the mutations were never transitory but remained fixed in the genomes. We have shown that loss of transmission was not due to lower viral titers in vesicular fluids; however, as we mentioned above, significant decreases in numbers and sizes of lesions may have impacted total viral yields from one to the next infectious round. In any case, progressive loss of virulence upon pig passages is related to viral genetic factors. These results suggest that if the observed loss of pathogenicity and

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TABLE 4. Rates of fixation of mutations and rates of O Tw97 evolution upon in vivo and in vitro passages

Passage group and infectious rounds	No. of substitutions per nucleotide per infectious round		Infectious round(s)[e,f]	O Tw97 evolution rate[c]
	Between passages[a,e,f]	Within the same passage[b,e,f]		
In vivo[d]				
T0/T1	3.7×10^{-4}		T0	0
T1/T1		6.2×10^{-4}	T1	3.7×10^{-4}
T1/T2	5.5×10^{-4}			
T2/T2		8.6×10^{-4}	T2	4.3×10^{-4}
T2/T3	4.6×10^{-4}			
T3/T3		2.5×10^{-4}	T3	2.9×10^{-4}
T3/T4	3.1×10^{-4}			
T4/T4		1.2×10^{-4}	T4	6.8×10^{-4}
T4/T5	1.2×10^{-4}			
T5/T5		2.5×10^{-4}	T5	7.4×10^{-4}
T5/T6	1.8×10^{-4}			
T6/T6		1.2×10^{-4}	T6	8.0×10^{-4}
T6/T7	6.1×10^{-4}			
T7/T7		6.2×10^{-4}	T7	8.0×10^{-4}
T7/T8	5.5×10^{-4}			
T8/T8		1.2×10^{-4}	T8	9.2×10^{-4}
T8/T9	3.1×10^{-4}			
T9/T9		4.9×10^{-4}	T9	9.8×10^{-4}
T9/T10	3.7×10^{-4}			
T10/T10		3.7×10^{-4}	T10	8.0×10^{-4}
T10/T11	2.1×10^{-4}			
T11/T11		3.7×10^{-4}	T11	1.0×10^{-3}
T11/T12	2.5×10^{-4}			
T12/T12		1.2×10^{-4}	T12	1.3×10^{-3}
T12/T13	4.3×10^{-4}	NA		
T13/T14	8.6×10^{-4}	NA	T13	1.7×10^{-3}
T14/T15	1.3×10^{-3}	NA	T14	1.8×10^{-3}
T15/T16	6.2×10^{-4}	NA	T15	7.4×10^{-4}
T16/T17	1.7×10^{-3}	NA	T16	1.2×10^{-3}
T17/T17		1.7×10^{-3}	T17	2.0×10^{-3}
T17/T18	1.4×10^{-3}			
T18/T18		4.9×10^{-4}	T18	1.6×10^{-3}
T18/T19	1.3×10^{-3}			
T19/T20	8×10^{-4}		T19	2.6×10^{-3}
T20/T20		8.6×10^{-4}		
T20/O Tw97			T20/O Tw97	2.4×10^{-3}
In vitro[d]				
A1/A3	1.2×10^{-4}		A1	0
A3/A6	0		A3	1.2×10^{-4}
A6/A9	0		A6	1.2×10^{-4}
A9/A12	0		A9	1.2×10^{-4}
A12/A15	0		A12	1.2×10^{-4}
A15/A18	1.2×10^{-4}		A15	1.2×10^{-4}
A18/A21	2.5×10^{-4}		A18	2.5×10^{-4}
A21/A23	2.5×10^{-4}		A21	4.9×10^{-4}
			A23	7.4×10^{-4}
B1/B3	1.2×10^{-4}		B1	0
B3/B6	0		B3	1.2×10^{-4}
B6/B9	2.5×10^{-4}		B6	1.2×10^{-4}
B9/B12	3.7×10^{-4}		B9	3.7×10^{-4}
B12/B15	2.3×10^{-3}		B12	7.4×10^{-4}
B15/B18	1.2×10^{-4}		B15	1.9×10^{-3}
B18/B21	0		B18	2.0×10^{-3}
B21/B23	0		B21	2.0×10^{-3}
			B23	2.0×10^{-3}
Lineages				
A1/B1	0			
A3/B3	0			
A6/B6	0			
A9/B9	2.5×10^{-4}			
A12/B12	6.2×10^{-4}			
A15/B15	1.7×10^{-3}			
A18/B18	1.7×10^{-3}			
A21/B21	1.7×10^{-3}			
A23/B23	1.4×10^{-3}			

[a] The number of substitutions per nucleotide per infectious round for one passage and its immediate predecessor was calculated as the average for four genomes compared by pairs.

[b] The number of substitutions per nucleotide for both replicates for each infectious round. NA, not available.

[c] The accumulative rate of incorporations of mutations during passages with T0 or A1 as the start point, respectively.

[d] Cell passage sequences are compared every three passages.

[e] The ranges (maximum and minimum numbers of changes detected during passages) are as follows: for between-pass rates in vivo, 1.3×10^{-3} to 8.0×10^{-4}; for between-pass rates in vitro, 1.2×10^{-4} to 2.3×10^{-3}; for O Tw97 evolution rates in vivo, 2.9×10^{-4} to 2.5×10^{-3}; for O Tw97 evolution rates in vitro, 1.2×10^{-4} to 2.0×10^{-3}.

[f] The averages (mean numbers of changes detected during the passages) are as follows: for between-pass rates in vivo, 6.4×10^{-4}; for between-pass rates in vitro, 1.4×10^{-4} (average B), 3×10^{-5} (average A), and 8.3×10^{-4} (average for lineages); for O Tw97 evolution rates in vivo, 1.3×10^{-4}; for O Tw97 rates in vitro, 4.0×10^{-4} (average B) and 9×10^{-5} (average A).

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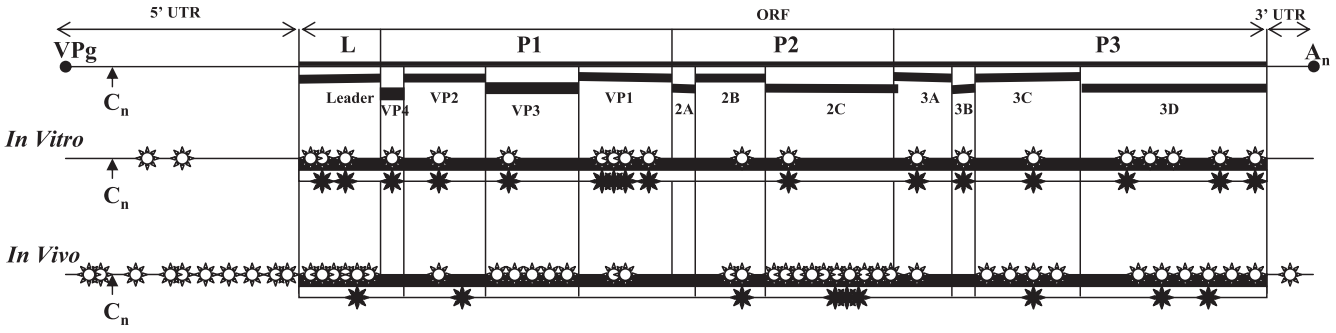

	In Vitro	*In Vivo*
lnL	-9347.722466	-9743.448951
Tree branch length	0.00496	0.02716
Ts/Tv rate ratio (K)	93.91067	20.95450
dN/dS rate ratio (ω)	1.7968	0.1669

FIG. 1. Schematic representation of changes observed in the FMDV genome during passages in vivo and in vitro. White stars represent nucleotide changes fixed along the genome during passages. Black stars represent nonsynonymous fixed nucleotide changes. Data summary of in vivo and in vitro parameters of selective pressure: ln*L*, neperian log likelihood ratio value; tree branch length, number of nucleotide substitutions per codon; Ts/Tv, transition/transversion; d_N/d_S, number of nonsynonymous substitutions per nonsynonymous site/number of synonymous substitutions per synonymous site.

accumulation of genetic mutations is due to the so-called Muller's ratchet effect, our data may reflect features of FMDV infection of great importance for pathogenesis, virus transmission, and FMD epidemiology in nature (7, 12, 13). In contrast to the observed resistance to extinction of FMDV subjected to plaque-to-plaque in vitro transfers (15, 25), animal-to-animal transmission in nature may lead to virus extinction. Under conditions of natural host transmission, a number of viral phenotypic functions are likely involved in virus-host interactions and an unknown number of bottlenecks lead to a continuous purification of the population, narrowing the mutant spectrum composition of the quasispecies in such a way that it is unable to successfully retain its fitness. This has recently been observed for another picornavirus, a poliovirus mutant displaying enhanced polymerase fidelity (35, 48).

For survival in nature, the viability, virulence, and transmission capability of FMDV must be maintained by as yet unknown mechanisms. In light of recent insights from bottlenecking effects observed during poliovirus infection in mice (36), it is likely that bottlenecking is the result of organ tropism and tissue-specific amplification within the host, resulting in the generalization of the progeny from very few particles of the parental quasispecies. Two observations suggest that more than one point of selection may act as a bottleneck during FMDV infection of the host. Our present data (Table 2) and previous reports have demonstrated with different FMDV isolates that 100 or more $TCID_{50}$ of BHK-21 cell passaged infectious viral particles injected i.d. into pigs result in identical parental and progeny viral consensus sequences (5, 6). The rapid imposition of new genetic variants observed here indicates that the initial route of animal infection imposes a serious barrier and acts as a bottleneck for the initial viral population. Additionally, it has been previously shown that an FMDV variant isolated during the febrile phase of the disease from blood from a pig infected with a highly purified homologous population of C-S8c1, an FMDV variant isolated from pigs and plaque purified three times in BHK-21 tissue culture before being inoculated into pigs (5), showed a consensus sequence different from that of the virus obtained from vesicles. This viremic variant was genetically stable upon cell culture and pig passages and showed phenotypic differences from the parental strain, which correlated with its origination from viremic blood (6). Thus, different viral variants cocirculate during FMDV infection of the host, likely as a result of bottlenecks during spread and replication within the host, although unless the genetic mutation selected during the bottleneck is advantageous with respect to the parental virus, the epitheliotropic FMDV consensus sequence will be the major progeny population in vesicular fluid. Therefore, more than one bottlenecking event may occur during FMDV infection and this may affect subsequent transmission in natural hosts.

The RasMol 2.7.1 program (www.rasmol.org) and published crystal structures of FMDV proteins were used to analyze predicted effects of amino acid changes detected in viral proteins during pig and cell culture passages. The E186/A substitution in L^{pro} falls in a highly disordered, unresolved region of the protease. The nonconservative Q580/R change affecting position 76 in VP3 is in close contact with P132 of the same protein, and its replacement by R may have an effect on the folding of the protein since R76 seems to interrupt the long α-helix structure of the parental sequence to induce a β-sheet structure. Secondary-structure analysis (Chou-Fasman) of the P114S substitution in the 3C viral proteinase predicts no significant effect. Both residues are small and uncharged; examination of the A10 virus 3C crystal structure revealed that P114 is on the protein surface, and although distant from the active

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TABLE 5. Amino acid changes observed following serial passage of O Tw97 in pigs

Pig designation	Infectious round	Amino acid for indicated polyprotein position, protein position, and viral protein[a]								
		PP 186, P 186, L^{pro}	PP 580, P 76, VP3	PP 987, P 34, 2B	PP 1139, P 32, 2C	PP 1242, P 135, 2C	PP 1408, P 301, 2C	PP 1753, P 105, 3C	PP 1863, P 11, 3D	PP 2008, P 156, 3D
Parental[b]	O Tw97	E	Q	N	A	T	I	P	E	K
48	T00v1									
48	T00v2									
48	T00v3									
50	T00v1									
50	T00v2									
50	T00v3									
483	T0									
484	T1									
485	T1									
486	T2									
487	T2									
488	T3									
489	T3									
490	T4			K						
491	T4			K						
492	T5			K						
493	T5			K						
494	T6			K						
495	T6			K						
496	T7									
497	T7									
498	T8									
499	T8									
725	T9									
726	T9									
4500	T10									
5008	T10						V			
5009	T11						V			
5010	T11						V			E
5011	T12						V			E
5012	T12						V			E
5013	T13				T	I	V			E
5014	T13						V			
5015	T14						V	S		E
4823	T15 (T14-I)						V	S	Q	E
4824	T16		R				V	S	Q	E
4825	T17		R				V	S	Q	E
4826	T17		R				V	S		
5184	T18		R				V	S	Q	E
5185	T18		R				V	S	Q	E
5005	T19 (T18-I)		R				V	S	Q	E
5006	T20	A	R				V	S	Q	E
5007	T20		R				V	S	Q	E
Conservation index		0.3	0.4	0.4	0.4	0.2	1.1	0.4	0.7	0.3
Protein Structure[c]		T to ND	α to β	T to α	α to β	None	None	None	None	ND to α
Charge[c]		− to N	N to +	N to +	None	None	None	None	− to N	+ to −

[a] Conservation indices (19) and predicted values of the detected amino acid substitutions. PP, polyprotein position; P, protein position.
[b] Parental refers to the expected amino acid present in the consensus sequence of parental virus O Tw97 that has been substituted in the following pig passages.
[c] T, turn; ND, not defined; α, alpha sheet; β, beta sheet.
[d] N, neutral; −, negative; +, positive.

site, P114 may somehow affect the protease substrate specificity pocket (S. Curry, personal communication). Substitutions in the 3D region, E11/Q and K156/E, seem to affect the protein surface and may have possible consequences for functional interaction between 3D and other proteins in the replication complex (Table 5).

The quantification of the FMDV genomic variability following the cell culture passage observed here is consistent with previous published reports (11, 16, 43, 44). Characterizations of FMDV genomic regions most extensively affected by mutations are difficult to reconcile with the many reports using VP1 as an indicator of variability to obtain phylogenetic information from field isolates (24, 26, 45). Previous analyses of partial sequences of the VP1-coding region following a single passage in vivo (5, 6), along with recent full-length genome studies performed with UK2001 field isolates, support our present

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data indicating that a surprisingly low number of mutations are found in SPs in animals that have not been vaccinated (9). These differences could be the result of early transmission events which precede development of antigenic variants due to host immune responses, while the field isolates compared in epidemiological studies come from animals with developing immune responses to previous infections and/or vaccination, which could act as driving forces for positive selection of antigenic variants (26, 34, 40).

Pigs 5017 and 4822 did not exhibit any sign of disease after 26 days in contact with T14 donors. Nevertheless, infectious virus was isolated from tonsil scrapings and nasal swabs collected from both animals, with titers of $10^{3.2}$ $TCID_{50}$/ml and $10^{3.9}$ $TCID_{50}$/ml and $10^{3.9}$ $TCID_{50}$/ml and $10^{2.4}$ $TCID_{50}$/ml, respectively. Virus isolation from pig tonsils at day 26 postcontact confirmed that these animals had been infected without clinical symptoms of disease and that virus had persisted in them for 4 weeks. This case resembles what has been described as a carrier state of FMDV for cattle, sheep, and goats (2, 39, 46) but not yet demonstrated for pigs. Bottleneck transmission may confer on the virus the ability to ratchet down fitness and virulence to ensure the immunization of at least a fraction of the population rather than kill or debilitate the entire susceptible host population. Indeed, most lineages would be destined to be self-limiting in subclinical and nonproductive infections. Interestingly, phylogenetically based epidemiological studies have indicated that FMDV topotypes appear to represent evolutionary cul-de-sacs (41). Our results suggest strong selection against changes in capsid proteins and higher flexibility for changes in NSP 2C and 3D in vivo, while a strong selection for substitutions in the P1 region (Table 3) is shown in vitro. These data confirm previous reports of spontaneous mutations in VP1 and the rise of antigenic variants occurring during FMDV replication in cell cultures in the absence of immunological selective pressure (11, 44). We do not understand this difference, since in both cases there is no immunological selective pressure. This observation may result from as yet unknown selective pressures involving viral receptor binding and/or particle internalization present in vivo. Finally, our results demonstrate that the effects of host adaptation can be objectively quantified and compared through the calculation of parameters of evolution and selective pressure, like those obtained with the CODELM analysis program (PALM). Although preliminary and limited, this is a novel and promising approach for analysis of FMDV genomic variability suggesting that the extension of our knowledge regarding viral evolution under experimental conditions in natural hosts will allow development of molecular epidemiology tools for improved identification of viral strains.

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